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14. ABSTRACT: The long non-coding RNA HOTAIR has been implicated as a critical regulator of breast cancer metastasis. HOTAIR is overexpressed in breast cancer, and can interact with the chromatin-modifier, Polycomb Repressive Complex 2, to initiate the silencing of important cancer progression genes. However, how HOTAIR localizes to and yields the silencing of specific genomic loci is unknown. We have developed new biochemical approaches to investigate the specificity of HOTAIR targeting in breast cancer cells. We have used quantitative mass spectrometry to identify the ensemble of proteins that associate with HOTAIR. We validated the interactions of candidate proteins with HOTAIR via Western blots and RNA immunoprecipitation. We also used a reconstituted chromatin *in vitro* system to evaluate recruitment of HOTAIR-interacting proteins to chromatin. To assess the functional significance of newly identified factors in HOTAIR-mediated gene silencing, we performed shRNA knockdown and then completed invasion and migration assays, in addition to chromatin immunoprecipitation to evaluate changes to histone modifications at HOTAIR target genes. Taken together, our experiments have identified a novel interaction partner of HOTAIR critical to its function. Through these studies, we will define the mechanism of HOTAIR-induced gene silencing and reveal how its aberrant expression promotes breast cancer metastasis.

15. SUBJECT TERMS
breast cancer, long non-coding RNAs, chromatin, metastasis, gene silencing

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INTRODUCTION:

The long non-coding RNA (lncRNA) HOTAIR has been implicated as a critical regulator of breast cancer metastasis. HOTAIR is frequently overexpressed in breast cancer and is associated with poor prognosis. HOTAIR interacts with a chromatin-modifying complex, Polycomb Repressive Complex 2 (PRC2), to promote the silencing of a subset of genes, including a number of cell-cell adhesion factors. Despite the important role of HOTAIR in cancer progression, the mechanism by which HOTAIR and PRC2 are targeted to specific genomic loci is unknown. Therefore, we have developed a series of biochemical and proteomic approaches to investigate the specificity of HOTAIR targeting in breast cancer cells. We discovered a strikingly specific interaction between the HOTAIR RNA and heterogeneous nuclear ribonucleoprotein (hnRNP) B1. Although B1 is involved in multiple aspects of mRNA processing, our experiments have demonstrated that B1 is also a critical component of the HOTAR-induced gene silencing machinery. Through a combination of *in vitro* and *in vivo* approaches, we have determined that B1 can engage HOTAIR on chromatin, and can modulate HOTAIR-induced silencing marks. By identifying key interaction partners of HOTAIR, we will define the mechanism of HOTAIR-induced gene silencing and reveal how aberrant expression of this lncRNA promotes breast cancer metastasis.

KEYWORDS: breast cancer, long non-coding RNAs, chromatin, metastasis, gene silencing

ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: Determine if HOTAIR is sufficient to initiate heterochromatin assembly from the silencing machinery of breast cancer cells

- We have completed Task 1a: Tether HOTAIR to a reconstituted chromatin assembly.
- We have completed 50% of Task 1b: Determine if an in vitro HOTAIR-chromatin assembly forms a heterochromatin domain.
- We have not yet completed Task 1c: Determine if HOTAIR expression in MDA-MB-231 cells is sufficient to assemble a heterochromatin domain.
- We have completed 75% of Task 1d: Test whether PRC2 components are recruited to HOTAIR-associated chromatin.

Aim 2: Test the hypothesis that HOTAIR recruits members of the larger PRC2 regulatory network, including NuRD and PRC1, which cooperatively initiate chromatin remodeling.

- We have completed Task 2a in months 1-12: Determine if members of the larger PRC2 regulatory network interact with HOTAIR RNA.
- We have completed 75% of Task 2b: Comparative mass spectrometry to characterize the HOTAIR-induced heterochromatin domain.
- We have completed 25% of Task 2c: Determine the requirements for HOTAIR-induced silencing *in vitro.*
- We have not begun Task 2d: Determine if the composition of HOTAIR-induced heterochromatin is conserved across different breast cancer cell lines.

Aim 3: Determine the components of the HOTAIR silencing machinery that drive breast cancer metastasis

- We have completed 50% of Task 3a: Use *in vitro* cellular invasion assays to identify the components necessary for HOTAIR-induced invasion (months 24-36).

- We have not yet completed Task 3b: Update pre-existing IACUC protocol A3269-01 to include our use of athymic mice as a model to investigate HOTAIR-induced breast cancer metastasis (months 18-24).
- We have not yet completed Task 3c: Determine the interaction partners of HOTAIR essential for metastasis in a xenograft mouse model (months 24-36).

What was accomplished under these goals?

In vitro identification of HOTAIR-interacting proteins

To identify HOTAIR-interacting proteins, we performed *in vitro* RNA pulldown experiments. We generated *in vitro* transcripts of full length HOTAIR or an antisense luciferase control lncRNA (Anti-Luc), which is of similar length and GC content as HOTAIR. The transcripts also included 10 tandem copies of a MS2 RNA aptamer at their 3' ends to allow purification of the RNA via MS2-Maltose binding protein (MS2-MBP) conjugated to amylose resin (**Figure 1A**). We incubated the tagged in vitro transcripts with nuclear extracts from HeLa or MDA-MB-231 cells, and purified proteins associating with the HOTAIR or the Anti-Luc lncRNAs and a no RNA control. qRT-PCR confirmed that our pulldown strategy consistently recovered ~40% of the HOTAIR RNA (**Figure 1B**). When we examined the associated proteins, we found that the HOTAIR transcript readily bound to its previously identified interaction partner, EZH2 (**Figure 1C**). However, EZH2 also bound to the Anti-Luc control RNA, and did not show specificity for HOTAIR (**Figure 1D**). These results are consistent with recent studies that revealed the promiscuous binding of EZH2 to numerous long RNA molecules(1). Therefore, additional components appear to be required for the specificity by which PRC2 is directed by HOTAIR to silence a subset of genomic loci.

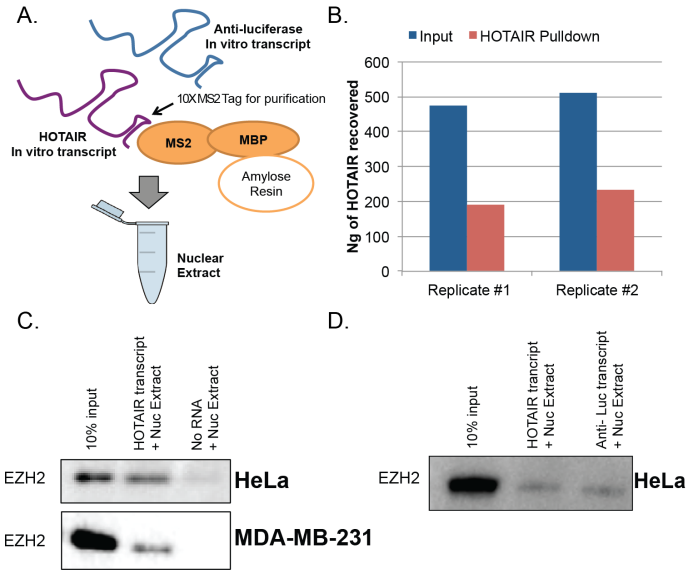

Figure 1. HOTAIR pulldown strategy. (A) Schematic of MS2-MBP purification strategy. (B) Recovery of HOTAIR IVT from pulldown by qRT-PCR. (C) Western blot to assess the enrichment of the PRC2 subunit, EZH2, with the HOTAIR IVT versus a no RNA control. (D) Western blot to compare EZH2 recovery by the HOTAIR versus an antisense-luciferase control lncRNA.

Quantitative mass spectrometry identifies the HOTAIR proteome

To comprehensively determine new interaction partners of HOTAIR that may govern this specificity, we performed comparative mass spectrometry following RNA pulldown (**Figure 2A**). We generated nuclear extracts from HeLa and MDA-MB-231 cells after performing stable isotope labeling of amino acids in cell culture (SILAC). By simultaneously assaying the proteins associated with HOTAIR or a lncRNA control with heavy labeled and normal nuclear extracts, we determined the proteins that specifically interact with HOTAIR. We identified multiple proteins enriched in the HOTAIR pulldown, including a number of heterogeneous nuclear ribonucleoproteins (hnRNPs) (**Figure 2B**). We validated the interactions of candidate proteins with HOTAIR via Western blots (**Figure 2C**), which revealed a very strong enrichment of the

hnRNP B1 protein with the HOTAIR transcript versus the Anti-Luc control.

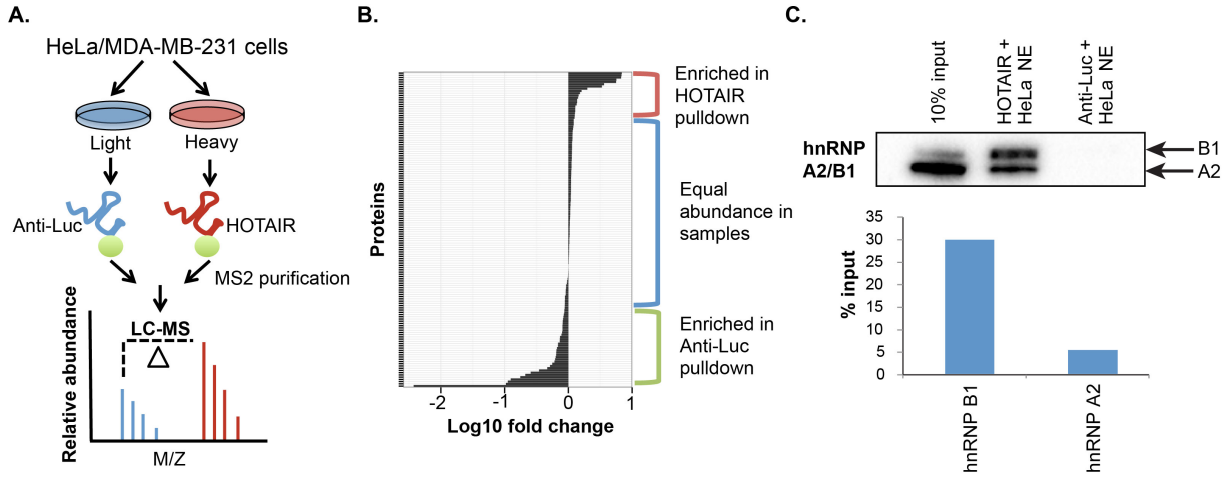

Figure 2. Purification of HOTAIR-associated proteins. (A) Stable isotope labeling of nuclear extract allows quantitative analysis of HOTAIR-enriched proteins. (B) LC/MS identifies subsets of proteins enriched in the HOTAIR vs. Anti-Luc pulldown. (C) Western blot confirms strong enrichment of hnRNP B1 with the HOTAIR transcript.

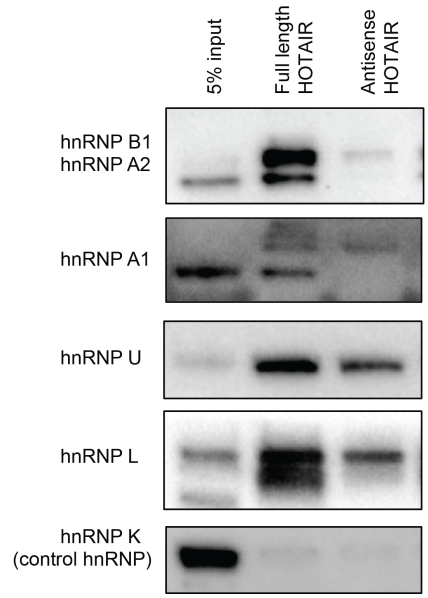

Figure 3. Western blot for HOTAIR-associated proteins.

To further confirm the specify of these interactions, we performed pulldowns using a tagged antisense version of the HOTAIR transcript, which revealed that the HOTAIR:hnRNPB1 interaction is unique for the HOTAIR sequence **(Figure 3)**. Western blots also validated the putative interactions between HOTAIR and two additional hnRNPs, hnRNP A1 and hnRNP L, identified by mass spectrometry. Importantly, HOTAIR did not interact with hnRNP K, which has previously been shown to interact with other lncRNAs and is highly abundant in our nuclear extracts. Therefore, our *in vitro* RNA pulldown assay captures the specific interactions between the HOTAIR lncRNA and a number of potential protein co-factors.

hnRNP B1 interacts with the HOTAIR lncRNA

We focused our efforts on the strikingly specific interaction between the HOTAIR RNA and hnRNP B1. B1 is a member of a large family of proteins involved in multiple aspects of mRNA processing. B1 is produced by the *HNRNPA2B1* gene and differs from its A2 isoform solely by the inclusion of an exon encoding 12-amino acids. hnRNPA2 and B1 have been implicated in directly modulating the transcription of specific genes, and to bind to and modulate the activity of the Cox2 lncRNA in immune cells **(2, 3)**. We found that the B1 isoform demonstrated a distinct preference for the HOTAIR RNA compared to A2 (**Figure 3**). High levels of the B1 isoform are known to be prognostic for early-stage breast cancer **(4)**, which suggests the B1 protein may serve as a critical regulator of HOTAIR-induced cancer progression.

Multiple domains of hnRNP B1 modulate HOTAIR binding

We next investigated the mechanism by which HOTAIR and hnRNP B1 interact. Previous work has demonstrated that different domains of HOTAIR show distinct binding affinities for certain chromatin modifiers. The catalytic component of PRC2, EZH2, has been shown to bind to the first 300 nt of HOTAIR **(5)**. Therefore, we constructed two truncated, MS2-tagged HOTAIR in vitro transcripts that contained either nucleotides (nt) 1-360 of HOTAIR or the remaining 360-2200 nt. The truncated and full-length HOTAIR transcripts were incubated with MDA-MB-231 nuclear extracts and purified via the MS2-MBP fusion protein. Following Western blot analysis of the associated proteins, we observed the greatest hnRNP B1 enrichment with full-length HOTAIR transcript compared to the two truncations (**Figure 4A**). Additionally, the 3' 360-2210 construct recovered much more hnRNP B1 than the short 5' 1-360 HOTAIR transcript. These results suggest that multiple regions of HOTAIR are required for the interaction with hnRNP B1, and the PRC2 binding site of HOTAIR is insufficient for B1 binding.

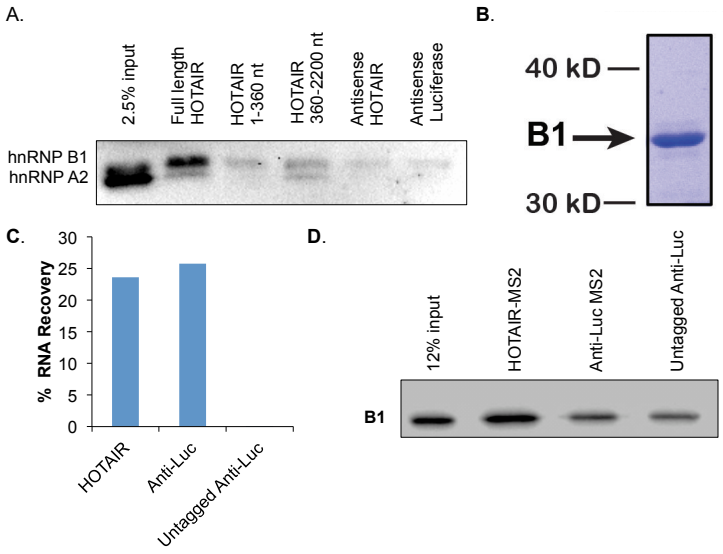

Figure 4. hnRNP B1 and HOTAIR directly interact. (A) Truncation analysis reveals that full length HOTAIR is required for strongest hnRNP B1 enrichment. (B) Commassie gel confirms production of recombinant hnRNP B1 protein. (C) In an RNA pulldown experiment with recombinant B1, equal quantities of the MS2-tagged HOTAIR versus Anti-Luc transcripts are recovered. (D) However, pulldown of HOTAIR yields strong enrichment for the hnRNP B1 recombinant protein by Western blot.

Direct interactions of hnRNP B1 and HOTAIR.

Because our RNA pulldown experiments were performed with nuclear extracts as the protein source, they raise the question of whether the hnRNP B1: HOTAIR interaction is direct, or if another protein or nucleic acid may be bridging the two molecules. To simplify the system and directly test hnRNP B1's ability to bind HOTAIR, we expressed and purified recombinant hnRNP B1 protein from *E. coli (***Figure 4B***)*. We then incubated the hnRNP B1 protein with the MS2-tagged HOTAIR transcript, and used the MS2-MBP fusion protein to purify HOTAIR and determine if hnRNPB1 associates with the RNA in the absence of other factors. We recovered equivalent amounts of the Anti-Luc and HOTAIR RNA (**Figure 4C**). However, Western blot demonstrated enrichment of hnRNP B1 in the HOTAIR pulldown compared to the MS2-tagged Anti-Luciferase and Untagged Anti-Luc controls, which demonstrates that hnRNP B1 directly binds the HOTAIR lncRNA (**Figure 4D**).

hnRNPB1 and HOTAIR interact in vivo

To expand our *in vitro* work to a more biological context, we next asked whether hnRNP B1 and HOTAIR interact endogenously within breast cancer cells. We generated MDA-MB-231 cells that stably overexpress HOTAIR cDNA by viral transduction, and performed native RNA immunoprecipitations (RIPs) of the hnRNP B1 protein using an antibody specific to the B1

isoform (**Figure 5A**). We used qRT-PCR to assess the recovery of RNAs from the B1 versus IgG antibody control RIPs. We found that B1 showed highly significant enrichment for the HOTAIR RNA versus the IgG control IP (p=0.002, t-test) (**Figure 5B**). As a control, we examined the recovery of the abundant U1 snRNA, which did not show substantial association with hnRNP B1. We also confirmed that in MDA-MB-231 cells that overexpress the Anti-Luc control lncRNA, immunoprecipitation of hnRNP B1 did not recover substantial amounts of Anti-Luc RNA (**Figure 5C**). Therefore, within breast cancer cells, hnRNP B1 demonstrate a specific and significant association with the HOTAIR lncRNA.

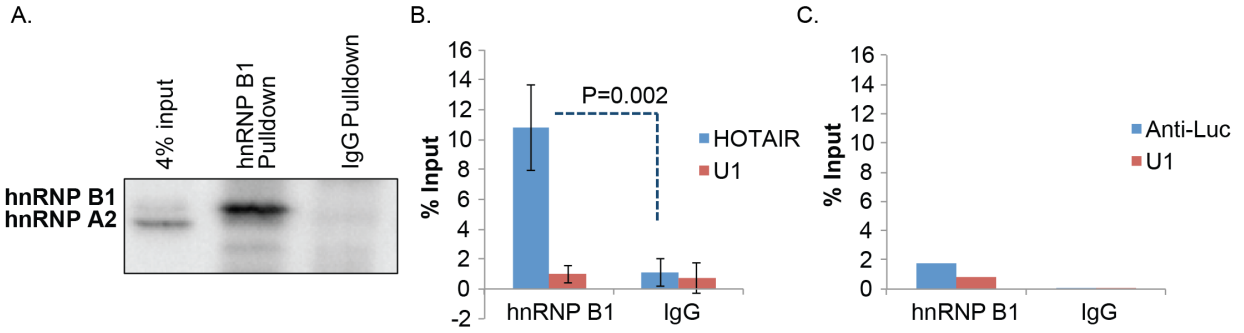

Figure 5. RNA immunoprecipitation of hnRNP B1 from MDA-MB-231 breast cancer cells. (A) Western blot confirms IP of the B1 isoform. (B) Recovery of the HOTAIR and U1 RNA by qRT-PCR following RIP of hnRNP B1 versus an IgG control in HOTAIR-overexpression cells. (C) Recovery of the Anti-Luc and U1 RNAs by qRT-PCR in Anti-Luc overexpression cells.

In vitro chromatin reconstitution of HOTAIR's interactions

We next sought to examine the interactions of hnRNPA2B1 and HOTAIR within the chromatin context. We used a reconstituted chromatin *in vitro* system to evaluate recruitment of HOTAIR-interacting proteins to chromatin. We generated nucleosome arrays containing human histone octamers as previously described [29]. Enzymatic assembly via the Nap1 histone chaperone and the ATP-dependent spacing complex, ISWI, was used to regularly space recombinant histones onto a 1.1 kb DNA substrate PCR product (**Figure 6**). The PCR template also contained six LexA binding sites, which can be bound by an RNA-binding protein fused to the LexA protein (**Figure 6A**). After testing multiple RNA binding proteins, the PP7 phage protein was determined to be the most efficient at tethering HOTAIR RNA, tagged with a tandem PP7 and tobramycin-binding aptamer (termed the RAT tag), to DNA and chromatin. We performed PCR with a biotinlyated primer in order to generate a chromatin template that could be conjugated

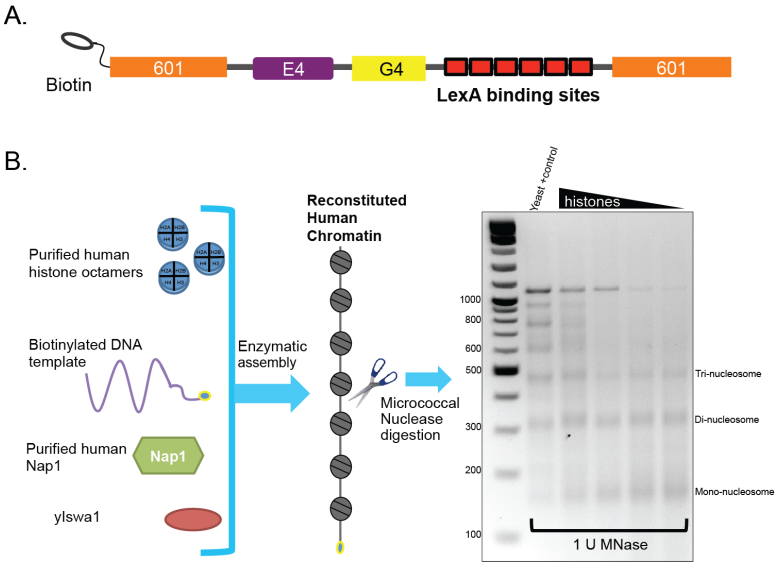

Figure 6. Chromatin in vitro reconstitution scheme. (A) DNA template for chromatin reconstitution. Six tandem LexA binding sites allow RNA tethering. (B) Reconstituted chromatin can be produced by enzymatic assembly and confirmed by micrococcal nuclease digestion.

to magnetic streptavidin beads for purification.

hnRNP B1 interacts with HOTAIR-tethered chromatin

We first tested whether our reconstituted chromatin could successfully tether HOTAIR. We incubated tagged HOTAIR in vitro transcript with chromatin bound by the recombinant PP7-LexA fusion protein. We found HOTAIR bound to the chromatin template only when both the recombinant PP7-LexA protein and its cognate LexA binding sites were present (data not shown). We next tested the ability of the HOTAIR-tethered chromatin to scaffold protein interactions. We assembled HOTAIR-bound chromatin onto magnetic beads, and incubated the assembly with nuclear extracts from HeLa and MDA-MB-231 cells. We then assessed protein and RNA recovery via Western blots and qRT-PCR respectively. As controls, we performed parallel experiments with a RAT-tagged Anti-Luc in vitro transcript, in addition to a naked DNA template that does not contain histones. We used the levels of H3 as a loading control to ensure that equal amounts of chromatin were included in each sample.

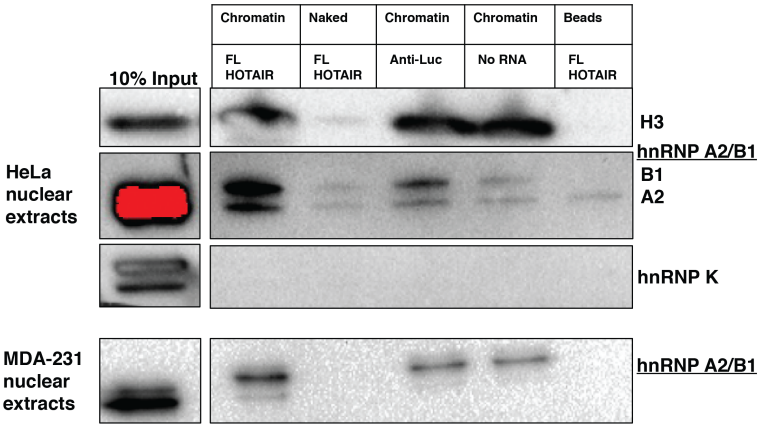

Figure 7. hnRNP B1 binds to chromatin in vitro in a HOTAIR-dependent manner. Western blot reveals the enrichment of hnRNP B1 on chromatin versus naked DNA. hnRNP B1 shows increased recruitment when the HOTAIR transcript is tethered to chromatin.

We found that chromatin promoted the recruitment of hnRNPB1 compared to the naked DNA. Importantly, we also observed that chromatin with HOTAIR present led to stronger enrichment of hnRNPB1. The B1 isoform showed much higher enrichment on chromatin than the more abundant A2 isoform, which again reveals the unique involvement of the minority isoform in HOTAIR and chromatin interactions. Taken together, our results show that B1 can engage HOTAIR on chromatin and suggest that hnRNP B1 may facilitate the targeting of the lncRNA silencing complex.

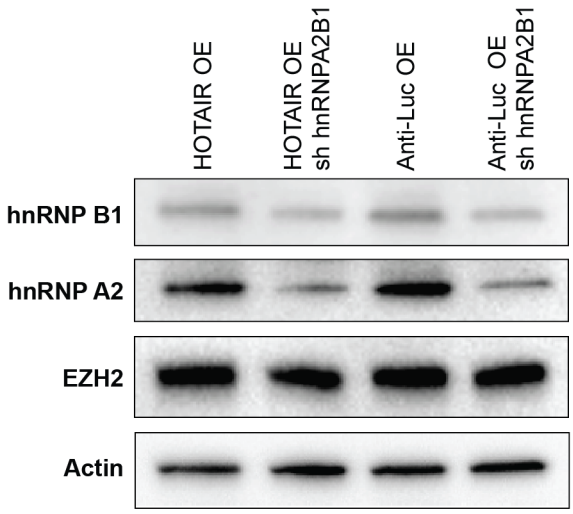

Figure 8. Knockdown of hnRNPA2B1 in MDA-MB-231 cells. Western blot confirms the knockdown of hnRNP B1 and A2 in breast cancer cells.

Knockdown of hnRNPAB1 affects gene expression

To assay the functional significance of this interaction, we generated MDA-MB-231 cells that overexpress HOTAIR or the Anti-Luc control and are silenced in their expression of *hnRNPA2B1*. Western blot confirmed successful knockdown of both isoforms (**Figure 8**). We next used microarrays and qRT-PCR to examine the gene expression changes resulting from hnRNPA2B1 knockdown. We found that widespread changes in gene expression following hnRNPA2B1 suppression, which is indicative of its regulation of multiple mRNA molecules in the cell. Importantly, hnRNPA2B1 knockdown led to an increase in a subset of genes enriched in a GO analysis as regulators of apoptosis ($p=0.04$). These results reinforce the

important role of hnRNP B1 in the survival of cancer cells. However, we observed that hnRNPA2B1 loss did not affect the protein levels of the PRC2 catalytic subunit EZH2. Microarrays performed on these cell lines further confirmed this result by showing that knockdown of hnRNPA2B1 did not lead to changes in the expression of the PRC2 subunits: EZH2, EZH1, SUZ12, JARID2, or AEPB2 (data not shown). These results are consistent with our model that hnRNPA2B1 affects HOTAIR directly, and does not regulate the expression of one of HOTAIR's protein co-factors.

hnRNPA2B1 is required for H3K27me3 at HOTAIR-target genes

We hypothesized that hnRNPB1 is required for the silencing activity of HOTAIR. To determine the importance hnRNPB1 in generating the H3K27me3 silencing mark, we performed chromatin immunoprecipitation in MDA-MB-231 cells +/- HOTAIR overexpression and +/- hnRNPA2B1 knockdown. We assessed the H3K27me3 and H3 levels at two previously identified HOTAIR target genes, HOXD10 and Junctional Adhesion Molecule 2 (JAM2). **Figure 9** depicts the ratio of H3K27me3 versus total H3 at each loci, which ensures that changes in nucleosome occupancy do not confound determination of specific changes in histone methylation. We found that suppression of hnRNPA2B1 led to a reduction in H3K27me3 in a HOTAIR-dependent manner. At the HOXD10 and JAM2 loci, knockdown of hnRNPA2B1 in HOTAIR overexpressing cells reduced H3K27me3 to the levels observed in cells expressing Anti-Luc. This effect is dependent upon HOTAIR, since Anti-Luc overexpression cells knocked down in hnRNPA2B1 showed similar H3K27me3 levels as the Anti-Luc parental line. These results demonstrate that hnRNPA2B1 is required for HOTAIR-induced chromatin modifications indicative of gene silencing.

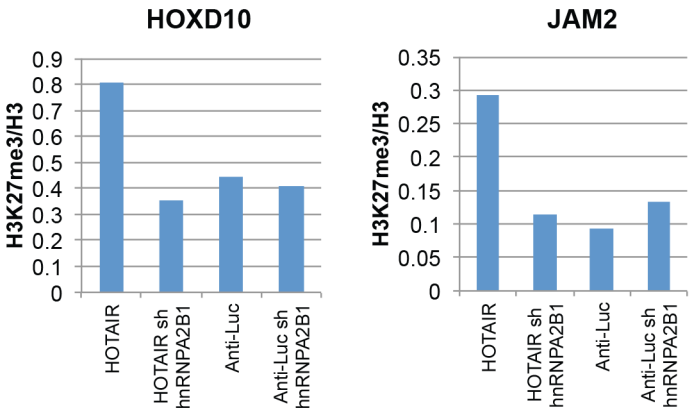

Figure 9. ChIP of H3K27me3 and H3 levels at HOTAIR-target genes. qRT-PCR was used to assess the enrichment of the HOXD10 and JAM2 loci in chromatin immunoprecipitations performed with H3K27me3 and H3 antibodies.

HOTAIR and hnRNPA2B1 regulate cell invasion

To determine the potential impact of hnRNPA2B1 and HOTAIR on cancer metastasis, we have performed preliminary in vitro invasion assays on MDA-MB-231 +/- hnRNPA2B1 and +/- HOTAIR or Anti-Luc overexpression. **Figure 10** shows three biological replicates for each cell line of invasion assays performed using cell culture inserts bearing 8 µm pores coated with Matrigel extracellular matrix. We found that suppression of hnRNPA2B1 in the HOTAIR overexpression line led to a reduction in invasion, and yielded similar levels of invasion as observed in the Anti-Luc overexpression lines +/- hnRNPA2B1 knockdown. Therefore, hnRNPA2B1 acts in a HOTAIR-dependent manner to modulate the invasive capability of breast cancer cells.

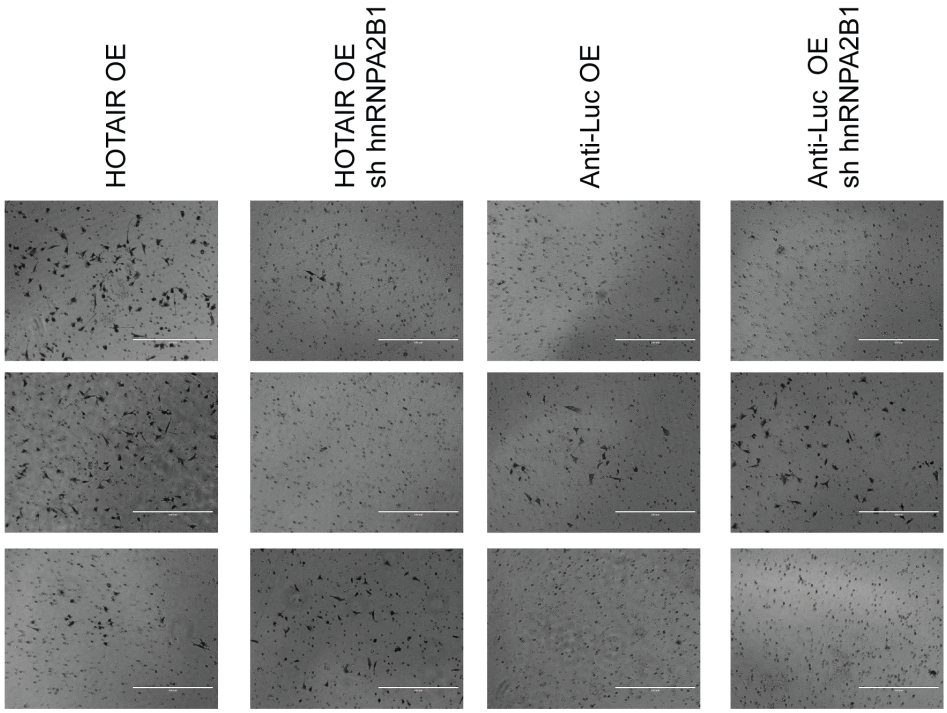

Figure 10. Invasion of MDA-MB-231 cells. The invasion of 100,000 MDA-MB-231 cells through Matrigel was quantified after 22 hrs, with three biological replicates for each cell line.

Summary of completed goals from project timeline

In our first year of work, we have identified hnRNP B1 as an important component in the mechanism of HOTAIR-induced gene silencing, and thus largely fulfilled our goals of Aim 2. We have also built a reconstituted chromatin assembly as described in Aim 1A, and begun to use this system to address how hnRNP B1 and HOTAIR affect recruitment of the silencing machinery to chromatin. Work from our lab and others have found the PRC2 components to show promiscuous binding to RNA in many experimental systems. Therefore, we will extend our work on Aims 1b, 1d, and 2a into the next year, and we will determine what factors control the endogenous specificity of PRC2. We have also begun Aim 3a earlier than planned, and have used *in vitro* invasion assays, in addition to chromatin immunoprecipitation, to examine the functional effects of hnRNP B1 on HOTAIR activity.

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What opportunities for training and professional development has the project provided?

Training opportunities

- I have received training in proteomic techniques from members of the UC Denver Proteomics Core, including Dr. Kirk Hansen and Dr. Monika Dzieciatkowska.
- I have learned protein expression and purification techniques from Dr. Aaron Johnson, including bacterial expression, ion exchange chromatography and affinity chromatography.
- I have received training in *in vitro* invasion and migration assays from members of Dr. Heide Ford's lab.

Professional development opportunities

- I completed a three-day long Career & Leadership Development workshop offered by the University of Colorado Graduate School as part of their NIH BEST Award.
- I have participated in career development training sessions offered through the University of Colorado Postdoctoral Office. Examples of these seminars included: Applying for academic jobs and Developing individual career development plans.
- I am registered to receive training in laboratory management in a day-long session on 10/16/14.
- I am enrolled in a 9-hr course on Responsible Conduct of Research Training offered throughout the academic year. I have attended the first seminar on Research Misconduct, and will continue to attend these monthly discussion sections for the next 8 months.
- I attended a Gordon Conference on Chromatin Structure and Function, and have participated in statewide conferences on Chromatin Biology.
- I have participated in the following seminar series offered on-campus: Chromatin Club (monthly), RNA Club (monthly), the Cancer Center Seminars (weekly), and the Biochemistry Departmental Seminars (weekly).

How were the results disseminated to communities of interest?

In the last year, I have presented my results at the following venues:

- Gordon Conference on Chromatin Structure and Function (poster at national meeting)
- The University of Colorado Comprehensive Cancer Center Annual Retreat, where I was awarded a "Best Poster" prize
- The Molecular Biology Department Annual Retreat (oral presentation)
- UC Denver Chromatin Club (oral presentation)
- Department of Biochemistry and Molecular Genetics weekly seminar (two oral presentations)

I have also served as a guest lecturer for undergraduates enrolled in a University of Colorado Denver course on Biotechnology. This experience allowed me to gain experience teaching, while also sharing my molecular biology expertise with a group of young scientists.

What do you plan to do during the next reporting period to accomplish the goals?

. In the next period, I plan to accomplish the following elements of my statement of work:

- I am currently preparing a manuscript to disseminate the results of my research.
- Complete Task 1b/d. We have already built an *in vitro* reconstituted chromatin system, which revealed that HOTAIR and chromatin engage hnRNP B1. We will next determine if this interaction leads to heterochromatin formation by probing for H3K27me3 by Western blot, and also evaluate the recruitment of the PRC2 complex.
- Complete Task 1c: Determine if HOTAIR expression in MDA-MB-231 cells is sufficient to assemble a heterochromatin domain. We have generated MDA-MB-231 cells overexpressing tagged and untagged HOTAIR. These cells will be used to purify HOTAIR and its associated proteins and determine if our in vitro chromatin template generates heterochromatin.
- Complete Task 2b. We have already performed comparative mass spectrometry on HOTAIR RNA pulldowns from nuclear extracts. We are currently expanding these studies to identify proteins bound to our chromatin assembly, in order to identify new HOTAIR regulators that act within the chromatin context.
- Begin Task 2c. We have purified recombinant PRC2, and will test if this complex is sufficient form heterochromatin on our reconstituted chromatin. Contributions of new factors such as hnRNP B1 will also be testing using purified proteins.
- Complete Task 2d: Determine if the composition of HOTAIR-induced heterochromatin is conserved across different breast cancer cell lines. I am currently expanding our work from HeLa and MDA-MB-231 cells to MCF-7 cells, which express higher endogenous levels of HOTAIR.
- Begin Task 3b. Update IACUC protocol.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

High levels of the long, non-coding RNA HOTAIR are associated with increased metastasis in breast cancer patients, but the underlying biological basis for its activity is not known. We have made substantial progress in determining the mechanism of HOTAIR. We have developed new biochemical tools that have allowed us identify candidate proteins that may regulate HOTAIR-mediated gene silencing and the promotion of cancer metastasis. Our proteomic techniques can be readily applied to other lncRNAs, in order to reveal the mechanisms of a poorly understood class of regulatory molecules with increasingly important roles in cancer. Our identification of hnRNP B1 as a critical element in HOTAIR-mediated silencing is noteworthy, as this protein has been reported to be a biomarker in breast and other cancers. We will expand this work by revealing the mechanism by which HOTAIR and B1 interact on chromatin to perpetuate gene silencing of cancer-relevant genes.

What was the impact on other disciplines?

Our work has clear clinical implications, as we are in the process of identifying promising therapeutic targets for restricting the oncogenic activity of HOTAIR. Currently, understanding of the HOTAIR molecular mechanism is too preliminary for direct application in patient care. We expect that our discovery of the interactions of HOTAIR and hnRNP B1 could serve as the basis for new therapeutic strategies. Important outcomes we expect to achieve are 1) determining how HOTAIR leads to aberrant gene silencing in cancer cells 2) defining the requirements for HOTAIR's promotion of breast cancer spread. By determining how HOTAIR leads to cancer spread, we hope to reduce the mortality of patients with metastatic breast cancer.

What was the impact on technology transfer?

- Nothing to report

What was the impact on society beyond science and technology?

- Nothing to report

CHANGES/PROBLEMS:

- **Changes in approach and reasons for change:** Nothing to report
- **Actual or anticipated problems or delays and actions or plans to resolve them:** Nothing to report
- **Changes that had a significant impact on expenditures**: Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**. Nothing to report.
- **Significant changes in use or care of human subjects**: Nothing to report
- **Significant changes in use or care of vertebrate animals**: Nothing to report
- **Significant changes in use of biohazards and/or select agents:** Nothing to report

PRODUCTS:

- **Publications, conference papers, and presentations**
  - Abstract presented at University of Colorado Comprehensive Cancer Center Annual Retreat, where I was awarded a "Best Poster Prize" for my work entitled, "The Mechanism of the Long Non-coding RNA HOTAIR in Breast Cancer"
  - Abstract presented at Gordon conference on Chromatin and Epigenetics. Presented poster entitled "Identification of the protein interaction partners of the long non-coding RNA HOTAIR"
- **Journal publications:** Nothing to report
- **Books or other non-periodical, one-time publications:** Nothing to Report
- **Other publications, conference papers, and presentations.**
  - The Molecular Biology Department Annual Retreat (oral presentation)
  - Department of Biochemistry and Molecular Genetics weekly seminar (oral presentation)
- **Website(s) or other Internet site(s):** Nothing to Report
- **Technologies or techniques:**
  We have developed a technique for using the MS2-RNA tag and the MS2-Maltose Binding protein fusion to isolate proteins that associate with the lncRNA HOTAIR in breast cancer cells. Following our publication of these data, we believe this technique can be applied to the study of many other lncRNAs critical in breast cancer progression.
- **Inventions, patent applications, and/or licenses:** Nothing to Report
- **Other Products:** Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	Emily Knouf Meredith
Project Role:	PI
Researcher Identifier (e.g.	0000-0001-8847-0346

ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Meredith has performed all of the experiments described above, with the exception of the in vitro chromatin reconstitution assays.
Funding Support:	

Name:	Maggie Balas
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Ms. Balas has developed the in vitro chromatin templates and performed the reconstitution assays
Funding Support:	CRNIC and NIH R00 Award to Aaron Johnson

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to Report

What other organizations were involved as partners? Nothing to Report

SPECIAL REPORTING REQUIREMENTS:

COLLABORATIVE AWARDS: Not applicable
QUAD CHARTS: Not applicable

APPENDICES:

University of Colorado Denver Cancer Center Abstract

Title: The Mechanism of the Long Non-coding RNA HOTAIR in Breast Cancer

Authors: Emily K Meredith, Maggie Balas, Heide Ford, and Aaron M Johnson.

Background: The long non-coding RNA (lncRNA) HOTAIR has been implicated as a critical regulator of breast cancer metastasis. HOTAIR is frequently overexpressed in breast cancer and is associated with poor prognosis. HOTAIR interacts with a chromatin-modifying complex, Polycomb Repressive Complex 2 (PRC2), to promote the silencing of a subset of genes, including a number of cell-cell adhesion factors. Despite the important role of HOTAIR in cancer progression, the mechanism by which HOTAIR and PRC2 are targeted to specific genomic loci is unknown. Therefore, we have developed a series of biochemical and proteomic approaches to investigate the specificity of HOTAIR targeting in breast cancer cells.

Materials and Methods: We have used quantitative mass spectrometry to identify the ensemble of proteins that associate with HOTAIR. We generated nuclear extracts from MDA-MB-231 breast cancer cells labeled with stable isotopes, and isolated the proteins interacting specifically with *in vitro* transcribed HOTAIR. Mass spectrometry identified multiple proteins enriched with HOTAIR versus a control lncRNA. We validated the interactions of candidate proteins with HOTAIR via Western blots and RNA immunoprecipitation. We also used a reconstituted chromatin *in vitro* system to evaluate recruitment of HOTAIR-interacting proteins to chromatin. To assess the functional significance of newly identified factors in HOTAIR-mediated gene silencing, we performed shRNA knockdown and then completed invasion and migration assays, in addition to chromatin immunoprecipitation to evaluate changes to histone modifications at HOTAIR target genes.

Results: We discovered a strikingly specific interaction between the HOTAIR RNA and heterogeneous nuclear ribonucleoprotein (hnRNP) B1. B1 is a member of a large family of proteins involved in multiple aspects of mRNA processing. B1 is produced by the HNRNPA2B1 gene and differs from its A2 isoform solely by the inclusion of an exon encoding 12-amino acids. Surprisingly, the B1 isoform demonstrated a distinct preference for the HOTAIR RNA compared to A2. High levels of the B1 isoform are known to be prognostic for early-stage breast cancer, which suggests the B1 protein may serve as a critical regulator of HOTAIR-induced cancer progression. We have demonstrated that recombinant hnRNP B1 directly binds to HOTAIR in the absence of other proteins. We also found that B1 can engage HOTAIR on chromatin, which suggests that hnRNP B1 may facilitate the targeting of the lncRNA silencing complex. Finally, to assay the functional significance of this interaction, we generated MDA-MB-231 cells that overexpress HOTAIR and are silenced in their expression of hnRNPA2B1. We found that suppression of hnRNPA2B1 led to a reduction in HOTAIR-induced silencing marks deposited on chromatin, which may potentially impact cellular gene expression patterns.

Conclusions: Taken together, these experiments expand the HOTAIR regulatory network to include the hnRNPB1 protein as a novel component of the gene silencing machinery. Misregulation of lncRNAs such as HOTAIR can dramatically reprogram the gene expression patterns of cancer cells. By identifying key interaction partners of HOTAIR, we will define the mechanism of HOTAIR-induced gene silencing and reveal how aberrant expression of this lncRNA promotes breast cancer metastasis.

Acknowledgements: Our work was supported by the DOD Postdoctoral Breast Cancer Fellowship to EM, and the NIH R00 Award to AJ.

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EDUCATION

University of Washington 2007-2012
Ph.D. in Molecular and Cellular Biology, GPA: 3.86

Pomona College 2003-2007
B.A. in Molecular Biology, *magna cum laude,* GPA: 3.94

RESEARCH EXPERIENCE

University of Colorado Denver, Dept. of Biochemistry 2013-present
Advisor: Dr. Aaron M Johnson
Postdoctoral Research: Mechanism of the long noncoding RNA HOTAIR in heterochromatin formation

- Determined the ensemble of proteins that associate with the HOTAIR RNA in cancer cells
- Investigated the proteins required for the promotion of cancer metastasis by HOTAIR

Fred Hutchinson Cancer Research Center, Dept. of Human Biology 2007-2012
Graduate Advisor: Dr. Muneesh Tewari
Dissertation: Regulation of microRNA expression and function in cancer

- Identified and characterized novel nucleotidyl transferase enzymes that regulate microRNA 3' nucleotide additions
- Characterized the regulation of miR-200 microRNAs by the p53 family of transcription factors in ovarian cancer
- Examined the extracellular communication of microRNAs through microvesicles

Pomona College, Dept. of Chemistry 2006-2007
Undergraduate Advisor: Dr. EJ Crane
Thesis: Identification of extremophile bacteria in hot spring environments

- Developed new methods for sample collection and used *16S* ribosomal sequencing to identify novel archaea and eubacteria residing in highly saline mud volcanoes

PUBLICATIONS

Chevillet JR, Kang Q, Ruf IK, Briggs HA, Vojtech LN, Hughes SM, Cheng HH, Arroyo JD, **Meredith EK**, Gallichotte EN, Pogosova-Agadjanyan EL, Morrissey C, Stirewalt DL, Hladik F, Yu EY, Higano CS, Tewari M. Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc Natl Acad Sci*. [Epub ahead of print] Sep 29 (2014).

Kullolli M, **Knouf E**, Arampatzidou M, Tewari M, Pitteri SJ. Intact MicroRNA Analysis Using High Resolution Mass Spectrometry. J Am Soc Mass Spectrom 25(1), 80-87 (2014).

Knouf EC, Wyman SK, Tewari M. The Human TUT1 Nucleotidyl Transferase as a Global Regulator of microRNA Abundance. *PLoS One* **8**, e69630 (2013).

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Simmons CS, **Knouf EC**, Tewari M, Lin LY. Utilization of plasmonic and photonic crystal nanostructures for enhanced micro- and nanoparticle manipulation. *J Vis Exp.* **55** (2011).

Wilson BK, Mentele T, Bachar S, **Knouf E**, Bendoraite A, Tewari M, Pun SH, Lin LY. Nanostructure-enhanced laser tweezers for efficient trapping and alignment of particles. *Optics Express* **18**, 16005-16013 (2010).

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Bendoraite A, **Knouf EC**, Garg KS, Parkin RK, Kroh EM, O'Briant KC, Ventura AP, Godwin A, Karlan BY, Drescher CW, Urban N, Knudsen BS, and Tewari M. Regulation of miR-200 family microRNAs and ZEB transcription factors in ovarian cancer: evidence supporting a mesenchymal-to-epithelial model of carcinogenesis. *Gynecol. Oncol.* **116**, 117-125 (2009).

* Indicates co-first authors

FELLOWSHIPS AND AWARDS

Department of Defense Postdoctoral Fellowship in Breast Cancer Research	2013-2016
University of Colorado Denver Cancer Center Best Poster Award	2014
Keystone Symposium Scholarship	2012
NIH NRSA Cellular and Molecular Biology Training Grant	2009-2012
National Science Foundation Graduate Fellowship, Honorable Mention	2008, 2009
Pomona College Walter Bertsch Prize in Molecular Biology	2007
Achievement Rewards for College Scientists Scholarship	2006-2007

CONFERENCE PRESENTATIONS

Poster: Gordon Conference on Chromatin Structure and Function 2014

- Title: Identification of the protein interaction partners of the long non-coding RNA HOTAIR**.** Waltham, MA

Selected speaker for short talk: Keystone Symposium on RNA Silencing 2012

- Title: Nucleotidyl transferases regulate the complexity and expression of the microRNA transcriptome. Vancouver, BC.

Poster: Keystone Symposium on MicroRNAs in Cancer 2011

- Title: Post-transcriptional generation of miRNA 3' variants. Banff, Alberta.

TEACHING EXPERIENCE

University of Colorado Denver Graduate School Orientation Instructor 2014

- Developed curriculum and led a new workshop to help incoming graduate

students create individual development plans for career advancement

University of Colorado Denver Guest Lecturer, Integrated Biology Dept. 2013
- Guest lecturer for undergraduate course on biotechnology

University of Colorado Denver Journal Club Discussion Leader 2013
- Led literature discussion sections for graduate students

University of Washington Teaching Assistant 2009
- UW Biology 355—Fundamentals of molecular biology: Independently led discussion sections for 50 undergraduate students once a week

Fred Hutchinson Science Education Partnership Mentor 2009
- Instructed high school science teachers in laboratory research and classroom lectures

Pomona College Laboratory Teaching Assistant 2004-2007
- Supervised students in the laboratory portions of General Chemistry, Organic Chemistry, Molecular Biology, and Biochemistry

SERVICE AND LEADERSHIP

Reviewer for Genes, Genomes, and Genetics 2014

University of Colorado Denver Postdoctoral Association Executive Committee 2013-present
- Secretary and Chair of Communications